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14. ABSTRACT

This research project focuses on the role of macrophages in early dissemination and dormancy. We hypothesized that macrophages are actively recruited by pre-malignant ErbB2 overexpressing cancer cells and that these intra-epithelial macrophages then produce factors that induce an EMT and thereby facilitate early dissemination. We further hypothesized that bone marrow (but not lung) macrophages produce TGFb2, BMP7 and other factors that instruct DCCs to enter dormancy. In this report we now provide evidence that early ErbB2+ lesions, but not healthy mammary tissue, produced CCL2 in an NFkB dependent manner and recruited intra-ductal macrophages, that secrete Wnt1 and thereby induce an EMT in the early ErbB2+ cancer cells. Depletion of macrophages, but only before overt advanced tumors appeared, drastically reduced early dissemination and surprisingly the onset of metastasis even after macrophages repopulated the overt tumor tissue. Importantly, humans with DCIS lesions, a very early stage of breast cancer, that contained macrophage+/E-Cadherin^{lo} microenvironments frequently had disseminated cancer cell (DCCs) in the bone marrow. We reveal that resident macrophages can promote early dissemination explaining how early cancer spread might proceed in breast cancer patients. We also propose that eDCCs play a long-term causal role in metastasis development.

15. SUBJECT TERMS

early dissemination, EMT, DCIS, macrophages, tumor microenvironment, metastasis

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1. INTRODUCTION

Most breast cancer patients die from metastatic disease that are mostly uncurable. Metastases can occur years or decades after removal of the primary tumor, suggesting there is a window of opportunity to prevent their outgrowth. Additionally, the field of metastasis research has been challenged by the finding that dissemination does not only occur from late stage invasive tumors but can already occur during early pre-invasive breast cancer stages as revealed by large cohort patient studies [1-4] and studies with spontaneous mouse tumor models [5]. This might lead to an increased heterogeneity of disseminated cancer cells (DCCs) that colonized target organs during different time points of progression and contribute to metastasis. Macrophages were implicated in regulating dissemination (local invasion migration and intravasation) in overt tumors with pathologically defined invasive characteristics [6, 7] and are important for growth of macro-metastases [7, 8], however whether they are involved in early dissemination and the regulation of growth of disseminated tumor cells has never been studied.

In this research project we **hypothesized** that macrophages are actively recruited by pre-malignant ErbB2 overexpressing cancer cells and that these intra-epithelial macrophages then produce factors that induce an EMT and thereby facilitate early dissemination. We further hypothesized that bone marrow (but not lung) macrophages produce TGFb2, BMP7 and other factors that instruct DCCs to enter dormancy.

In this report **we now provide evidence** that early ErbB2+ lesions, but not healthy mammary tissue, produced CCL2 in an NFkB dependent manner and recruited intra-ductal macrophages, that secrete Wnt1 and thereby induce an EMT in the early ErbB2+ cancer cells. Depletion of macrophages before overt advanced tumors appeared drastically reduced early dissemination and surprisingly, the onset of metastasis even after macrophages repopulated the overt tumor tissue. Importantly, humans with DCIS lesions, a very early stage of breast cancer, that contained macrophage+/E-Cadherin^{lo} microenvironments frequently had disseminated cancer cell (DCCs) in the bone marrow. We reveal that resident macrophages can promote early dissemination explaining how early cancer spread might proceed in breast cancer patients. We also propose that eDCCs play a long-term causal role in metastasis development.

For the next two funding years, **we are planning** to further address the question how macrophages interact with disseminated tumor cells once these reach a distant organ and specifically, elucidate on the mechanisms on how bone marrow macrophages instruct dormancy whereas lung macrophages induce tumor cell proliferation.

2. KEY WORDS

early dissemination, EMT, DCIS, macrophages, tumor microenvironment, metastasis

3. ABBREVIATIONS AND NOMENCLATURE

We previously referred to cells in very early stage breast cancer lesions as "pre-malignant epithelial cells" (PM-MECs). In the manuscript we changed this nomenclature to "early cancer cells" (eCCs) to address the possibility that there might be a subpopulation of invasive cancer cells within early lesions. Consequently, we refer to "disseminated cancer cells" (DCCs). In the manuscript we also use the synonym Her2 instead of ErbB2. However this is the same protein and mouse model.

EMT – epithelial to mesenchymal transition
DCIS – ductal carcinoma in situ
CC – cancer cell
eCC – early cancer cell
CCC – circulating cancer cell
DCC – disseminated cancer cell

4. ACCOMPLISHMENTS.

Specific Aim 1. To determine the role of MΦs in early dissemination.

Objectives. We will determine which cytokines produced by ErbB2^{hi} / p38^{low} mammary ducts recruit MΦs. We will investigate how intra-epithelial MΦ induce an EMT in ECCs. We will characterize a profile of an early dissemination microenvironment that we will confirm using 3D *in vitro* experiment. We will further confirm the significance of our findings using human tissue microarrays to identify those genes whose expression indicate an early dissemination microenvironment.

Results. All experiments suggested in SA1 have been successfully performed and will be submitted as a research paper to the journal Cell this month (September 2015). The manuscript is attached. Results are summarized below with references to the attached manuscript figures.

We determined the macrophage recruiting cytokines as follows: We analyzed the expression of the suggested factors (CSF-1, CSF-2, CCL-2, IL-1b, IL-6) in mammospheres derived from pre-malignant MMTV-ErbB2 mice and found upregulation of CCL-2 and CSF-2 on the mRNA level (manuscript Fig.3A). We then confirmed upregulation of CCL2 on the protein level by immunofluorescent staining of FVB wild type and pre-malignant MMTV-ErbB2 mammary gland sections (manuscript Fig.3B-E). We next identified that CCL2 upregulation in pre-malignant epithelial cells was dependent on ErbB2 mediated induction of NFkB as evidenced by reduction of phosphorylation of the p65 subunit of NFkB when pre-malignant mammospheres were treated with the ErbB2 inhibitor lapatinib (manuscript Fig.3F) and reduction of CCL2 immunofluorescent signals in pre-malignant mammary acini when ErbB2 or NFkB signaling was blocked (manuscript Fig.3G-I). To functionally validate these findings, early cancer cell (eCC) acini were treated with inhibitors against ErbB2, NFkB, or CCL2 signaling and primary mammary tissue macrophages were added. We found that inhibition of the ErbB2 – NFkB – CCL2 signaling axis led to reduction in the recruitment of macrophages to acini (manuscript Fig.3J-M). When pre-malignant MMTV-ErbB2 mice were treated with an inhibitor against the CCL2 receptor CCR2 for 2 week in vivo, the number of intra-epithelial macrophages was inhibited significantly (manuscript Fig.3O-Q).

We characterized a profile of an early dissemination microenvironment as follows: To analyze Wnt proteins upregulated in intra-epithelial macrophages, Raw264.7 macrophages were treated with conditioned medium from either FVB wild type or pre-malignant MMTV-ErbB2 mammospheres. We then performed RealTime PCR analysis for the suggested Wnt candidates (Wnt-1A, Wnt-3A, Wnt5A, Wnt7) and found significant upregulation after MMTV-ErbB2 CM treatment only of Wnt1 (manuscript Fig.4I). We confirmed these findings

with primary mammary tissue macrophages isolated from pre-malignant MMTV-ErbB2 mice that were treated with the same conditioned medium (manuscript Fig.4H). To test whether Wnts derived from macrophages indeed influenced EMT of mammary epithelial cells, we performed co-culture experiments and added the Wnt inhibitor DKK1. In line with our hypothesis, we found that addition of macrophages to Comma-1D monolayers induced strong down regulation of E-Cadherin whereas addition of DKK1 rescued this macrophage-induced EMT (manuscript Fig.4J-L).

We confirm the significance of our findings using human DCIS patient material with known DCC status: Since all of our experiments gave us robust and cohesive results, we decided to skip the validation of our findings in commercially available tissue microarrays where it is unknown whether these patients carried disseminated tumor cells. Instead, we received 20 DCIS section from Dr. Tanja Fehm, a collaborator of the mentor (University Clinic Duesseldorf, Germany). Blindly, we stained those sections for E-Cadherin and CD-68, a human macrophage marker and then predicted the DCC status as follows: patients who carried lesions with low E-Cadherin signals and high number of intra-epithelial macrophages were considered high risk whereas patients with high E-Cadherin signals and low number of intra-epithelial macrophages were considered low risk (manuscript Fig.6A,B). Based on these criteria, we predicted that 10 patients would be positive for DCCs and 10 patients would be negative for DCC. After unblinding, we found that our positive prediction was right in 70% of the cases (manuscript Fig.6C). The results did not reach statistical analysis due to the small sample size but it is a promising result that strengthens our finding that macrophages drive dissemination and indicates that analysis of tumor microenvironments of early dissemination could be used as a diagnostic tools to identify women at high risk of developing metastatic disease.

Additional findings: To test whether early dissemination can also contribute to metastasis formation eventually, we blocked CSF-1Ra during early and asymptomatic stages of cancer, starting at age week 18, and stopped as soon as tumors became palpable. Then we waited until tumors reached 1 cm in diameter (4-6 weeks later) and quantified DCC and metastatic lesion burden (manuscript Fig.6D). We found that the time to tumor detection as well as the time it took until these tumors were overt was not affected by CSF-1Ra blockade during early cancer stages (manuscript, supplementary Fig.4A,B). Additionally, histologic features and macrophage content in contra-lateral mammary glands as well as the invasive tumors and vascularization of tumors at the end of the experiment were comparable in control and in anti-CSF-1Ra treated mice (manuscript, supplementary Fig.4D-G). However, CSF-1Ra blockade during early stages of cancer had a profound effect on DCC burden (manuscript Fig.6E) and late metastasis development (manuscript Fig.6F) even after macrophage depletion had been stopped for one month in average and animals had carried fast-growing and large invasive tumors. The number of solitary DCCs in late stage animals, likely to be a mixture of DCCs accumulated since the early stages, was reduced by CSF-1Ra blockade during early stages of cancer. This suggests that the reduced influx of DCCs to lungs during early stages was not replaced by DCCs arriving during the time of tumor detection to euthanasia, a period where large tumors with invasive phenotype grew. Importantly, metachronous micro-metastases were reduced when macrophages had been depleted during early cancer stages. We interpret that macrophages by playing a seminal role in early dissemination, allow for the early target organ colonization by a sub-population of eDCCs that have metastasis initiating capacity or aid the formation of metastatic breast cancer by DCCs that arrive later during progression.

Specific Aim 2: To determine how target organ-specific MΦ dictate DCC fate.

Objectives. To determine how lung resident MΦs induce a proliferation program and how BM resident MΦs induce a dormancy program in DCCs. We will compare expression profiles of lung and BM resident MΦs and compare expression levels of dormancy inducing factors previously described in our lab (i.e. TGFβ2, BMP7). We will validate identified factors responsible for the induction of a dormancy program *in vitro* and *in vivo*.

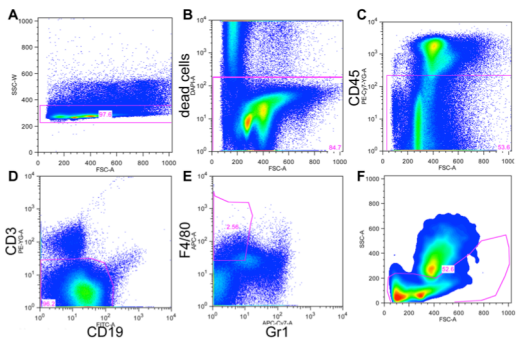

Fig.2.1: Gating strategy for bone marrow macrophages. After gating out doublets (A), dead cells (B), non hematopoietic cells (C) and T- and B-cells (D), F4/80+ and Gr1- cells were identified (D) and SSChi eosinophils were gated out (E).

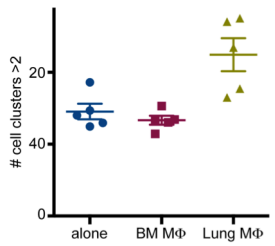

Fig.2.2: 3D co-culture of 4T1 cells with tissue resident macrophages. 4T1-GFP cells were either seeded alone or mixed with primary lung or bone marrow macrophages. Dividing 4T1 cells (clusters >2 cells) were quantified after 48h.

Testing the effect of lung and BM MΦs on tumor cell proliferation: To start isolating bone marrow and lung macrophages, we tested FACS staining protocols and optimized staining for bone marrow resident macrophages. We decided to not use CD115 as a marker since further preliminary experiments showed that the antigen is frequently internalized and thus not as reliable for FACS sorting (data not shown). Instead we also gated out non T-cells and non B-cells (CD3, CD19) and then proceeded with our established gating protocol as described in the original proposal (gate out Gr1+ granulocytes, gate out SSC^{hi} eosinophils, isolate F4/80+ macrophages) (Fig.2.1). This gave us a higher purity of bone marrow resident macrophages. Additionally, we found that the MMTV-ErbB2-CFP cell line available in our lab tends to enter senescence after several rounds of cultivating. We therefore decided to use established and commercially available cell lines in the meantime. To this end, we started experiments using the 4T1 mammary carcinoma cell line tagged with GFP or mCherry. We performed a co-culture experiment where macrophages were sorted from bone marrow and lungs of wild type mice. 4T1-GFP cells were seeded in matrigel either alone or with bone marrow or lung macrophages in a ratio of 1:1. After 48h, we quantified the number of 4T1-GFP clusters of two or more cells that were derived from proliferating 4T1 cells. We found that lung macrophages had a significant growth stimulatory effect on 4T1-GFP cells whereas bone marrow macrophages tended to inhibit cancer cell growth (Fig.2.2). This confirms the findings presented in the original proposal, where we found that bone marrow conditioned medium inhibited MMTV-ErbB2 cancer cell growth and that this can be reversed when macrophages are depleted whereas the opposite is true for lung conditioned medium. Importantly, this also provides first evidence that it is a direct effect of bone marrow versus lung macrophages that decide over the growth fate of disseminated cancer cells, as we had hypothesized. We are currently further evaluating this experiment and performing experiments using macrophage conditioned medium to complete the proposed experiment for SA2.1.1.

Expression profile of target organ resident MΦs and testing of MΦ-derived dormancy inducing factors: As stated in the original statement of work, these experiments proposed in SA2.1.2 and 2.1.3 were planned for funding year two and three.

Training opportunities. The training opportunities according to the statement of work were met; these training opportunities were: sorting of mammary gland macrophages; performing RealTime PCRs, performing 2D and 3D in vitro cultures, use combinatory pharmacologic inhibitors. Additionally, meetings with the Co-mentor Dr. Merad and Dr. Aguirre-Ghiso were held as well as with the mentoring committee to discuss progress of the project.

Dissemination of results to the community. The work presented here is part of a research manuscript that is being submitted to Cell this week will be publicly available soon. Additionally, the results have been presented in several conferences (see Product section).

Plans for the next funding period. We successfully performed all suggested experiments of Specific Aim 1 and thus fulfilled our time plan for the statement of work as submitted in the original application ahead of time. For Specific Aim 2, we optimized the experimental approach, performed parts of the experiments suggested in SA2.1.1 and are currently performing the remaining experiments planned for funding year 1. As proposed in the original statement of work, expression profiling of target organ resident macrophages will be performed in funding year two and testing of macrophage derived dormancy inducing factors will be performed in funding year two and three.

5. IMPACT

Approximately 90% of breast cancer patients die from commonly incurable metastases. Metastases can occur years or decades after removal of the primary tumor, suggesting there is a window of opportunity to prevent their outgrowth. Additionally, recent clinical data demonstrating that dissemination can occur much earlier than assumed and that patients carrying pathologically defined pre-invasive breast cancer lesions (e.g. ductal carcinoma in situ, DCIS) can carry dormant DCCs. This has caused great confusion on how to treat women with early stage breast cancer such as DCIS. A recent study published in JAMA Oncology [9] adds to this confusion as it shows that while breast cancer deaths from DCIS are rare, 50% of those cases occur in the absence of an invasive breast cancer recurrence and that the choice of therapy did not affect survival. This indicates that albeit at early frequency, a subpopulation of women with DCIS carry early disseminated cancer cells that can have deadly consequences. We therefore need better tools to identify those DCIS patients at high risk of developing late metastatic relapses without overtreating the majority of women who have harmless variants of DCIS. Our mechanistic work on early dissemination that we were able to carry out with the support of this DoD breast cancer award has led us to the notion that those DCIS lesions carrying an E-Cadherin low/macrophage high signature might be indicative of the presence of disseminated disease.

6. CHANGES/PROBLEMS

No major changes or problems.

7. PRODUCTS

Manuscripts:

„Macrophages orchestrate early dissemination of HER2+ cancer cells.“ Nina Linde, Arthur Mortha, Nicole Saenger, Maria Soledad Sosa, Ethan Tardio, Tanja Fehm, Thomas Karn, Miriam Merad, and Julio A. Aguirre-Ghiso. – Manuscript will be submitted to Cell in week of September 21, 2015.

Oral Presentations:

„Macrophages orchestrate early metastatic dissemination of pre-malignant ErbB2+ mammary epithelial cells“ Cancer Biology Retreat, Mount Sinai, New York, NY, December 2014

Poster presentations:

„Macrophages orchestrate early metastatic dissemination of pre-malignant ErbB2+ mammary epithelial cells“ Annual Meeting of the American Society of Cell Biology, Philadelphia, PA, December 2014

„Macrophages orchestrate early metastatic dissemination of pre-malignant ErbB2+ mammary epithelial cells“ Cancer Cell Symposium on Cancer Inflammation, Sitges, Spain, June 2015

Awards:

Icahn School of Medicine at Mount Sinai Postdoctoral Recognition Award, New York, December 2014

8. PARTICIPANTS

no change

9. LITERATURE

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10. APPENDIX: Manuscript on the following pages

Macrophages orchestrate early dissemination of HER2+ cancer cells.

Nina Linde[1], Arthur Mortha[2], Nicole Saenger[3], Maria Soledad Sosa[1], Ethan Tardio[1], Tanja Fehm[4], Thomas Karn[3], Miriam Merad[2], and Julio A. Aguirre-Ghiso[1,5].

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Summary

Metastasis dissemination can already occur during early pre-invasive breast cancer stages. However mechanisms that control early cancer cell dissemination are unclear. Here we show that MMTV-HER2 early cancer lesions contain an invasive subpopulation of HER2+/E-cadherin^{lo} cancer cells that disseminate by recruiting resident CD206+ mammary tissue resident macrophages (MTMs). This occurs months before monocyte-derived macrophages are recruited into advanced tumors. Early HER2+ lesions, but not healthy mammary tissue, produced CCL2 in an NFkB dependent manner and recruited intra-ductal MTMs, that induced an EMT in the early HER2+ cancer cells. Depletion of MTMs, but only before overt advanced tumors appeared, drastically reduced early dissemination and surprisingly the onset of metastasis even after macrophages repopulated the overt tumor tissue. Importantly, humans with DCIS lesions that contained macrophage+/E-Cadherin^{lo} microenvironments frequently had disseminated cancer cell (DCCs) in the bone marrow. We reveal that resident MTMs can promote early dissemination explaining how early cancer spread might proceed in breast cancer patients. We also propose that eDCCs play a long-term causal role in metastasis development.

Introduction

The field of metastasis research has been challenged by the finding that dissemination does not only occur from late stage invasive tumors but can already occur during early pre-invasive breast cancer stages as revealed by large cohort patient studies (Banys et al., 2012; Braun et al., 2005; Sanger et al., 2011; Schardt et al., 2005) and studies with spontaneous mouse tumor models (Husemann et al., 2008). This might lead to an increased heterogeneity of disseminated cancer cells (DCCs) that colonized target organs during different time points of progression and contribute to metastasis. We follow the early and late DCCs definition by (Husemann et al., 2008) where "early DCCs" (eDCCs) originate at times when patients are thought to carry only *in situ* (e.g. DCIS) lesions and "late DCCs" are derived from invasive tumors. Early dissemination occurs in spontaneous mouse models of breast cancer such as the MMTV-HER2 and -PyMT models (Husemann et al., 2008) and in pancreatic cancer models (Rhim et al., 2012). In the MMTV-HER2 model, early-disseminated cancer cells were able to form lung metastases in the absence of invasive carcinoma (Husemann et al., 2008). This argued that in these models eDCCs are endowed with latent metastasis initiating capacity. Similarly, in some cases patients with DCIS can develop breast cancer metastases even without developing local invasive breast cancer (Cutuli et al., 2001; Donker et al., 2013; Narod S.A., 2015; Narod et al., 2015; Warnberg et al., 2001; Warnberg et al., 2014). This might indicate that, albeit at low frequency, eDCCs can form metastases also in patients. However, how early cancer cells disseminate and how they contribute to metastasis is unclear. This scenario is further complicated by the current paradigm that states that dissemination and metastasis is associated with advanced aggressive tumors that acquire invasive mechanisms. However, the occurrence of early dissemination (Banys et al., 2012; Husemann et al., 2008) and other studies of epithelial cell motility (Liu et al., 2015; Nguyen-Ngoc et al., 2012) argue that multiple genetic alterations might not be needed for dissemination from early primary lesions. Thus, cancer cells from early progressed lesions may use epigenetic and micro-environmental mechanisms to turn on an invasive phenotype. If this is correct, what are the mechanisms that drive early dissemination and do eDCCs have an impact on metastasis development later in progression?

Macrophages were implicated in regulating dissemination (local invasion, migration, and intravasation) in overt tumors with pathologically defined invasive characteristics (Lin et al., 2001; Qian et al., 2009). However whether macrophages participate in early dissemination has not been studied. Macrophages are powerful drivers of intravasation and dissemination from invasive breast cancer tumors *via* the establishment of tumor microenvironments of metastasis, or TMEM, where a specialized macrophage regulates entry into the vasculature (Harney et al., 2015). This follows a streaming process where first, breast tumor cells recruit macrophages *via* CSF-1 and second, cancer

cell motility is stimulated via macrophage-derived EGF (Wyckoff et al., 2004). Additionally, macrophages can induce an epithelial to mesenchymal transition (EMT) in malignant cells. (Bonde et al., 2012; Liu et al., 2013). However, macrophages could be operational during earlier stages of cancer because they orchestrate invasion and migration of mammary epithelial cells during normal mammary tree branching morphogenesis into the fat pad (Gouon-Evans et al., 2000). We hypothesized that early cancer cells might be dependent on stromal cells for invasion and that mammary tissue macrophages (MTMs) may be recruited much earlier than predicted to propel invasive events. We further hypothesized that this may be the consequence of early aberrant signals triggered by oncogene signaling.

Here we show that stromal resident mammary tissue macrophages (MTM) are recruited into the epithelial layer of early mammary cancer lesions defined as ADH or DCIS-like. This process depends on HER2 activity and NFkB-mediated induction of CCL-2. The intra-epithelial macrophages disrupt the myoepithelial layer locally and then induce an epithelial to mesenchymal transition (EMT)-like response in a subpopulation of HER2+ cancer cells. This results in active intravasation and early dissemination to the lung that could be efficiently blocked by macrophage depletion. Depletion of macrophages for a limited timeframe in the asymptomatic phase also reduced lung metastatic burden during later stages of invasive tumor growth when animals carried large primary tumors. Our results suggest a previously unrecognized role for MTMs in favoring dissemination of HER2+ tumor cells much earlier than expected and and a role for eDCCs in late metastasis. Since metastatic cancer is incurable and the present understanding how early dissemination contributes to metastasis is limited this might also lead to important clinical considerations.

RESULTS

MACROPHAGES INFILTRATE THE EPITHELIAL COMPARTMENT OF EARLY BREAST CANCER LESIONS.

We compared macrophages in healthy and pre-malignant human breast tissue. We found that in breast tissue from healthy donors, macrophages are localized in the stroma in vicinity to mammary ducts but remain outside of the ducts which are delimited by an intact myoepithelial layer of cells (**Fig.1A**). In contrast, even in DCIS lesions that displayed an apparently intact myoepithelial layer, there was a statistically significant increase in the frequency of macrophages found inside the aberrant ductal epithelial structure in between cancer cells (**Fig.1B,C**). Next, we analyzed the localization of macrophages in pre-malignant stages in the MMTV-HER2 murine model of breast cancer that shows slow progression, allowing for detailed analysis of early lesions such as ADH and DCIS. We stained mammary gland sections of MMTV-HER2 mice for the murine macrophage marker F4/80. Like in human healthy tissue, macrophages occupied the stroma outside of mammary ducts in healthy FVB wild type animals (**Fig.1D**). This was also true in young 14 week old MMTV-HER2 mice

(**Fig.1E**). However, when MMTV-HER2 mice progressed over time but showed yet no signs of palpable tumors (18-24 weeks), macrophages were often localized inside the pre-malignant mammary luminal epithelial layer as demonstrated by co-staining of F4/80 and cytokeratin 8/18 (**Fig. 1F**). We hypothesized that as macrophages enter the epithelial layer, they might disrupt the architecture of the duct. Close inspection of sections co-stained for smooth muscle actin and F4/80 showed that the myoepithelial cell layer was frequently disrupted at sites where macrophages were in immediate contact with the duct (**Fig. 1G-I**). Quantification of the abundance of intra-epithelial macrophages confirmed that the incidence of ducts with intra-epithelial macrophages depended on HER2 upregulation and pre-malignant disease progression (**Fig. 1L**). Intra-epithelial macrophages could also be detected using the macrophage marker Iba1 and were positive for CD206, a marker for M2 polarization in macrophages (Fig.1J-L). Interestingly macrophages found inside late stage invasive tumors of MMTV-Her2 showed lower CD206 levels than pre-malignant intra-epithelial macrophages (Fig.1M-O).

INTRA-EPITHELIAL MACROPHAGES IN EARLY LESIONS ARE TIE2+ M2-POLARIZED MAMMARY TISSUE MACROPHAGES.

We next used mass cytometry (CyTOF) to investigate the myeloid cell populations in early cancer lesions. Mammary glands derived from FVB wild type mice, 14 and 22 week old pre-malignant and invasive overt tumors from MMTV-HER2 mice were analyzed using CyTOF with a panel of 17 hematopoietic cell markers (**Supplementary Fig. 1A**). To determine the identity of the intra-epithelial macrophages and distinguish between resident mammary tissue macrophages (MTMs) that regulate mammary gland development, involution and tissue homeostasis (Angela C. L. Chua, 2010; Gouon-Evans et al., 2000) or monocyte-derived tumor-associated macrophages (henceforth termed TAMs) we used 17 markers to identify accurately myeloid lineage cells and we then detected MTMs as CD206+ and TAMs as VCAM+ as recently described (Franklin et al., 2014) (gating strategy in **Supplementary Fig. 1B)**. In agreement with Franklin et al., 2014, we found that healthy wild type mammary glands contained CD206+ M2 polarized MTMs and that VCAM+ TAMs were predominantly present in invasive tumors where they constituted the majority of all myeloid cells (**Fig.2B,C, supplementary Figure 2B,C**). MTMs but not TAMs were predominantly Tie2+ (**Fig.2D, supplementary figure 2D**) and CD11c- (**Fig.2E**) and $CD86^{hi}$ and $CD11b^{hi}$ (**Fig.2F,G**) and thus resembled Tie2+ or TMEM macrophages known to drive dissemination (Harney et al., 2015; Pucci et al., 2009). Interestingly, TAM content in early lesions in MMTV-Her2 mice was not increased significantly but they contained mostly MTMs (**Fig.2A, supplementary figure 1C**) and their intra-epithelial macrophages were CD206+ (**Fig.1J,K**), indicating that intra-epithelial macrophages in pre-malignant stages are MTMs and not monocyte-derived TAMs and that these share a profile with

TMEM macrophages (Harney et al., 2015) and which are recruited into the duct upon HER2 upregulation.

HER2+ EARLY CANCER CELLS PRODUCE CCL2 AND RECRUIT MACROPHAGES INTO MAMMARY DUCTS.

We next explored whether Her2+ cancer cells from early lesions were capable of recruiting macrophages through cytokine production. We isolated early cancer cells from mammospheres derived from either FVB wild type or MMTV-HER2 early cancer lesions as described (Cohn et al., 2010) and performed quantitative real-time PCR analysis for cytokine mRNAs known to recruit macrophages into overt tumors. Surprisingly we found that already at these early stages of progression HER2+ cancer cells significantly upregulated CCL-2 and CSF-2 (**Fig. 3A, supplementary figure 3A**) but not CSF-1, IL-1β, and IL-6 (**supplementary Fig3A**). Upregulation of CCL-2 but not of CSF-2 was also observed at the protein levels also as revealed by immunofluorescence (**Fig.3B-E and data not shown**). In invasive breast cancer models HER2 signaling activates NFkB, which transcriptionally induces CCL-2 (Cogswell et al., 2000). Accordingly, the p65 subunit of NFκB subunit was phosphorylated in mammospheres derived from early MMTV-HER2 cancer cells and its phosphorylation was inhibited by Lapatinib, a HER2 inhibitor (**Fig.3F**). Additionally, acinar-like structures produced by early MMTV-HER2 cancer cells displayed a reduction in CCL2 production upon inhibition of HER2 or NFkB signaling with specific inhibitors (Basseres et al., 2014) (**Fig.3G-I**). To confirm that CCL-2 signaling was necessary and sufficient for HER2-dependent macrophage recruitment, HER2+ early cancer cells were grown as 3D acinar structures *in vitro* for 5 days. Cultures were then treated with Lapatinib, an IKKβ inhibitor (Basseres et al., 2014) or an inhibitor of the CCL2 receptor CCR2 (RS504393) and macrophages isolated from mammary glands of MMTV-HER2 mice were added (**Fig.3J-M**) to the cultures. After 24 hours of co-culture, macrophages were associated with ~50% of all acini in control samples whereas co-cultures treated with the inhibitors all showed significant reduction in macrophage association (**Fig.3N**). We next treated 20 week old MMTV-HER2 mice carrying only early cancer lesions (no palpable or overt tumors) for 2 weeks with a CCR2 inhibitor (**Fig.3O,P**). We found that the number of intra-epithelial macrophages was significantly reduced when mice were treated with the CCR2 inhibitor (**Fig.3Q**). MTMs can be depleted by genetic ablation of CCR2 (Franklin et al., 2014) arguing that we may be eliminating the same population during early mammary cancer stages. We conclude that HER2 signaling in early cancer cells activates NFkB to induce CCL2, which in turn recruits CCR2+ macrophages into the early lesions.

MACROPHAGES INDUCE AN EMT-LIKE RESPONSE IN EARLY HER2+ CANCER CELLS.

We investigated whether HER2 might aberrantly activate a mechanism of invasion and motility involving macrophages and an EMT already in pathologically-defined pre-invasive lesions. Strikingly we found that intraepithelial macrophages induced a strong local down regulation of E-cadherin junctions *in vivo* in early cancer cells during stages of pre-invasive cancer when they were located directly adjacent to them (1-2 cell diameter away) (**Fig.4A-C**). This was paralleled by a general downregulation of E-Cadherin mRNA in 20 week old mammary glands compared to wild type tissue (**Fig.4D**). A loss of E-Cadherin and translocation of beta-Catenin to the nucleus could also be modeled *in vitro* when Raw264.7 macrophages were added to Comma-1D healthy mammary epithelial cell monolayers (**Fig.4E-G**). The loss of E-cadherin junctions in intra-epithelial cells adjacent to macrophages suggested that macrophages might produce cues that stimulate an EMT. Since the EMT induction was local, and macrophages can produce Wnt ligands (Cosin-Roger et al., 2013; Wang et al., 2015), we hypothesized that macrophages might secrete hydrophobic Wnt proteins known to be potent EMT inducers. Raw264.7 macrophages or MTMs isolated from pre-malignant MMTV-HER2 mammary glands were treated with conditioned media from normal epithelial cells from FVB wild type mice or from early mammary cancer cells from MMTV-HER2 mice. Only conditioned media derived from early HER2+ mammary cancer cells induced an upregulation in the expression of Wnt-1 (**Fig.4H, I**), which is also produced by TAMs (Ojalvo et al., 2010) in macrophages but not Wnt-3, Wnt5a and Wnt7 that did not change or were not expressed (**Supplementary Fig.4A**). The loss of E-Cadherin junctions in Comma-1D mammary epithelial cells induced by addition of Raw264.7 macrophages could be rescued when DKK1, an inhibitor of Wnt signaling, was added to co-cultures (**Fig.4J-M**). These results suggest that upon recruitment, the close association of early HER2+ cancer cells with macrophages results in an EMT-like response in the early cancer cells possibly propelled by Wnt signaling.

The association of intra-epithelial macrophages with reduced E-Cadherin levels was confirmed in human DCIS samples. It was independent of HER2 status and it appeared in different patterns. Patients with high macrophage numbers within lesions showed overall lower E-Cadherin levels as measured by fluorescence image analysis **(Fig.4N-P**). Additionally, within the same patient, individual lesions with high macrophage numbers had lower E-Cadherin levels (**supplementary Fig.4B-D**). This reveals an inter- and intra-tumor heterogeneity and suggests that some regions of DCIS lesions might be more prone to produce early epithelial cancer cells with an EMT like program and invasive and disseminating capacity.

MACROPHAGE DEPLETION PREVENTS AN EMT AND EARLY DISSEMINATION.

Our data indicated that early in HER2-driven cancer evolution, macrophages are actively recruited into early cancer lesions where they induce an EMT in early cancer cells. We hypothesized that this could in turn lead to early dissemination. To directly confirm this, we depleted macrophages from pre-malignant MMTV-HER2 mice with the ASF98 antibody against CSF-1Rα (**Supplementary Fig.5A**). CSF-1Rα blockade led to efficient depletion of $CD11b^{+}/F4/80^{+}/Gr1^{-}$ macrophages. (**supplementary Fig**.**5B**). Immunofluorescence staining for F4/80 in HER2+ early lesions confirmed a significant reduction in the number of intra-epithelial macrophages when CSF-1Rα was blocked (**supplementary Fig.5C**). This was accompanied by a significant reduction in the number of hyperplastic ducts (**supplementary Fig.5D**) and a tissue wide upregulation of E-Cadherin expression (**Fig.5A**) and E-cadherin-based junctions throughout the mammary tree (**Fig.5B,C**). Quantification of E-cadherin intensity showed that E-cadherin upregulation occurred to levels far higher than those even seen in the early lesions of control treated MMTV-HER2 mice, independently of the location of macrophages (**Fig. 5D**). Disruption of the myo-epithelial layer evidenced by loss of coverage of early cancer cells by myo-epithelial cells in the basal plane was strongly reduced upon CSF-1Rα blockade (**Fig.5E-G**). We conclude that when in contact with early HER2+ cancer cells macrophages contribute locally to the acquisition of properties of invasive tumor cells. We further conclude that macrophages recruited by cancer cells in early lesions negatively influence E-cadherin junction formation and expression.

The early cancer cell EMT-like response caused by the crosstalk with macrophages led us to test whether this relationship caused dissemination. To this end we quantified early circulating cancer cells (eCCCs) detected by cytokeratin 8/18 and HER2 positive staining in peripheral blood harvested from MMTV-HER2 mice. CSF-1Rα blockade significantly reduced the numbers of eCCCs, suggesting an active role for these myeloid cells in early dissemination (**Fig.5H**). To test whether CSF-1Rα blockade also reduced eDCC burden in target organs, we stained lung sections for surface HER2 expression and found that CSF-1Rα blockade significantly decreased the number of eDCCs in the lungs (**Fig.5I-K**). We conclude that eCCs recruit CD206+ MTMs to acquire an invasive and disseminating phenotype which results in the accumulation of disseminated cancer cell in target organs early in cancer progression.

PATIENTS WITH $E\text{-CADHERIN}^{LO}/MACROPHAGE^{+}$ DCIS LESIONS MORE FREQUENTLY DISPLAY BONE MARROW ASPIRATES POSITIVE FOR DCCS.

Banys et al., demonstrated that about 13% of patients with DCIS had detectable DCCs in their bone marrow (BM), but neither histologic markers nor receptor status were indicative of the presence

of DCCs (Banys et al., 2012). To test whether the presence of intra-lesion macrophages may indicate early dissemination, we performed a pilot study in 20 primary DCIS samples. In a blinded approach we analyzed E-cadherin levels and macrophage presence in DCIS samples with known status for the presence of BM DCCs. We next grouped those with low E-Cadherin signals and high numbers of intra-epithelial macrophages as high risk candidates for carrying DCCs (**Fig.6A,B**). Based on this analysis, we could identify DCIS patients with BM DCCs with an accuracy of 70% (**Fig.6C**) by looking at the presence of an E-Cadherin^{lo}/macrophage^{+} profile in their primary lesions. While the small available sample size does not allow to thoroughly test statistical significance these data and that in Fig.4 strongly suggests that the analysis of macrophages and EMT markers in the surrounding tumor cells might be useful to identify patients who may already have early disseminated disease.

MACROPHAGE ORCHESTRATED EARLY DISSEMINATION CONTRIBUTES TO METACHRONOUS METASTASIS FORMATION.

While early cancer lesions can spawn a population of eDCCs with metastatic capacity (Hosseini et al., 2015 submitted), it is not clear whether the eDCC with metastasis initiating capacity are those aided to disseminate by macrophages. To test this possibility we blocked CSF-1Rα during early and asymptomatic stages of cancer, starting at age week 18, and stopped as soon as tumors became palpable. Then we waited until tumors reached 1 cm in diameter (4-6 weeks later) and quantified DCC and metastatic lesion burden (**Fig.6D)**. We found that the time to tumor detection as well as the time it took until these tumors were overt was not affected by CSF-1Rα blockade during early cancer stages (**supplementary Fig.6A,B**). Additionally, histologic features and macrophage content in contra-lateral mammary glands as well as the invasive tumors and vascularization of tumors at the end of the experiment were comparable in control and in anti-CSF-1Rα treated mice (**supplementary Fig.6C-G**). However, CSF-1Rα blockade during early stages of cancer had a profound effect on DCC burden (**Fig.6E**) and late metastasis development (**Fig.6F**) even after macrophage depletion had been stopped for one month in average and animals had carried fast-growing and large invasive tumors. The number of solitary DCCs in late stage animals, likely to be a mixture of DCCs accumulated since the early stages, was reduced by CSF-1Rα blockade during early stages of cancer. This suggests that the reduced influx of DCCs to lungs during early stages was not replaced by DCCs arriving during the time of tumor detection to euthanasia, a period where large tumors with invasive phenotype grew. Importantly, metachronous micro-metastases were reduced when macrophages had been depleted during early cancer stages. We interpret that macrophages by playing a seminal role in early dissemination, allow for the early target organ

colonization by a sub-population of eDCCs that have metastasis initiating capacity or aid the formation of metastatic breast cancer by DCCs that arrive later during progression.

Discussion.

Considerable evidence (Banys et al., 2012; Braun et al., 2005; Husemann et al., 2008; Schardt et al., 2005) supports the hypothesis that metastasis can be initiated by DCCs early in cancer progression. However, the cancer cell intrinsic and microenvironmental mechanisms responsible for early dissemination remained unclear. Here we reveal a previously unappreciated mechanism by which early cancer cells in the MMTV-HER2 model recruit macrophages, which in turn induce an EMT–like response in early cancer cell dissemination and metastasis.

Our studies provide several key findings worth discussing. The role of macrophages in early breast cancer progression has been understudied possibly because most models focus on overt invasive cancer lesions. Accordingly, these studies adhere to the notion that the stromal to epithelial barrier is intact in early breast cancer lesions deemed non-invasive by pathological analysis (Park et al., 2010). However, micro-invasion events were detected in patient lesions using electron microscopy and these may go unnoticed by light microscopy (Husemann et al., 2008). The link of macrophages to metastasis was identified in the primary tumor where CSF-1 produced by the cancer cells recruit CSF1Rα+ macrophages that in turn produce EGF to stimulate motility and intravasation of cancer cells (Wyckoff et al., 2004). More recently, powerful intravital imaging reveled that a F4/80+/CD206+/Tie2+/VEGF+ subpopulation of TAMs reside in tumor microenvironments of metastasis (TMEM), where they serve as exclusive portals for intravasation (Harney et al., 2015). Further, metastasis-associated macrophages (MAMs) that are recruited by micro-metastasis in a CCL2- but also CCL3-dependent manner to promote metastasis (Kitamura et al., 2015; Qian et al., 2011). Finally, in overt tumors TAMs and not MTMs prevent a proper T-cell anti-tumor response (Franklin et al., 2014). Because these studies did not focus on early stages of cancer the identity and function of macrophages at work went unnoticed. We reveal that already during early stages of mammary cancer, macrophages are already functional to regulate dissemination. These M2 polarized CSF1Rα+/ CD206+/V-CAM- macrophages have the same profile of the TMEM macrophages that are gatekeepers of the intravasation doorways (Harney et al., 2015). This function was proven when we found that depletion of macrophages in early HER2+ lesions using anti-CSF1Rα antibodies, reversed in early HER2+ cancer cells, the EMT-like response intravasation and dissemination to lungs. Additional studies from our lab (Harper and Sosa et al., 2015 submitted) show that cancer cell intrinsic HER2-dependent induction of Wnt signaling induced an EMT-like program that along with the help of MTMs guarantees efficient early dissemination. The specific contribution of MTMs vs. TAMs,

that are also present although in less proportion in the early lesions, needs further scrutiny. However, that V-CAM+ macrophages were not present in the population of intraepithelial macrophages during early stages of mammary cancer argues that MTMs might be the functional subtype during this stage. Further, whether in early lesions macrophages regulate the streaming of cancer cells (Wyckoff et al., 2004), TMEM formation (Harney et al., 2015) or T-cell regulation (Franklin et al., 2014), is still unclear. That TAMs are recruited later suggests that this increase in macrophages may fulfill additional functions perhaps related to immune suppression (Franklin et al., 2014), promotion of proliferation and vascularization, among others (Joyce and Pollard, 2009). Our results here show that MTM-like macrophages are rate limiting for early dissemination and thus reveal that MTMs might be as powerful as TAMs in regulating EMT-like programs fueling early dissemination.

Our results appear to have a correlate in human breast cancer, as DCIS lesions had at high frequency elevated levels of intra-epithelial macrophages that produced cancer cell microenvironments low for E-cadherin. In addition DCIS, lesions that had high frequency of intra-epithelial macrophages and reduced E-Cadherin levels associated with the presence of bone marrow DCCs. Because, all commonly used predictive markers had failed to indicate the presence of DCCs in DCIS patients (Banys et al., 2012), our studies strongly support larger patient studies to support that these mechsnisms might be at work in human lesions considered for the most part non-invasive and low risk.

It was unclear whether the eDCCs that are stimulated to intravasate by the MTMs contributed to metastasis. It is often argued that while 13% of DCIS patients show some form of disseminated disease and only 3% develop metastatic disease (Cutuli et al., 2001; Donker et al., 2013; Narod et al., 2015; Warnberg et al., 2001; Warnberg et al., 2014), early dissemination is not a contributor to lethal metastatic cancer. However, approximately 50% of breast cancer deaths after DCIS occurred in the absence of a local invasive recurrence and was not influenced by current treatments(Narod et al., 2015). Thus, while at lower frequency, women carrying eDCCs can develop lethal metastases (Narod et al., 2015). In addition, eDCCs may cooperate with later arriving DCCs to form metastasis in patients that after DCIS treatment go on to develop invasive lesions or in patients that had DCIS but only were diagnosed later for invasive cancer. Such information is difficult to gather and functionalize with human samples. However, our experiments showed that macrophages are rate limiting for early dissemination and also metastasis formation. This is because macrophage depletion before overt tumor formation, was sufficient to significantly reduce solitary DCC and metastatic burden. This was true even after TAMs had massively infiltrated overt tumors. These findings challenge the view that eDCCs might be innocuous and rather on the contrary indicates that they critically contribute to the development of metastases through biology that we have not grasped. Our current model (**Fig.6**) proposes that eDCCs might directly seed metastasis as supported by pioneering studies (Husemann

et al., 2008) and our recent studies where early cancer cells activate an EMT-like response and form metastasis (Hosseini et al., 2015 submitted and Harper and Sosa et al., 2015 submitted). In addition, eDCCs might also prime the target organ microenvironment for DCCs that arrive later making it permissive for growth. This resembles and does not exclude the concept of the “pre-metastatic” niche described previously (Kaplan et al., 2005; Peinado et al., 2012). However, in our theory the pre-metastatic niche might be orchestrated by eDCCs even after primary tumor surgery.

We provide critical new insight into the understanding of the natural history of metastatic disease by discovering that MTMs propel early dissemination and that combined quantification of macrophages and E-cadherin might provide a tool to identify patients with DCIS that are at risk of already carrying disseminated disease. Most importantly, we demonstrated that eDCCs appear to play a seminal role in metastatic breast cancer. These findings warrant further studies to understanding the role of eDCCs that persist over time in target organs and how they contribute to metastasis. This information is critical to target the full heterogeneity of disseminated disease and prevent metastasis development.

Figure legends

Figure 1: Macrophages enter the ductal epithelial layer in early breast cancer lesions. (A) Human adjacent healthy (A) and DCIS tissue (B) was stained against CD68 (macrophages) and smooth muscle actin (SMA) and the percentage of ducts in each patient sample containing intra-epithelial macrophages (IEM) was determined (C). Mammary glands from FVB wild type (D) of pre-malignant MMTV-HER2 mice at age 14 (E) and 22 (F) weeks were stained against F4/80 (macrophages) and CK8/18 (epithelial cells) and against F4/80 and SMA (G-I). The percentage of ducts containing IEM for in individual animals was quantified (J). Mammary glands from MMTV-Her2 mice at age 22 (J-L) and invasive tumors at age 26 (M-O) were stained against Iba1 and CD206.

Figure 2: Phenotypic profiling of macrophages in early mammary cancer lesions. Whole mammary glands from FVB wild type mice or 14 and 20 week old pre-malignant MMTV-HER2 mice and invasive tumors from MMTV-HER2 mice were analyzed by mass cytometry. Visne plots were generated from myeloid cells (gating strategy supplementary Fig.1AB) were divided into Ly6C+ monocytes and F4/80+/CD64+ macrophages (A). Macrophages were differentiated into CD206+ mammary tissue macrophages (MTMs) and VCAM+ tumor associated macrophages (TAMs). Two individual animals per group were analyzed (top and bottom row). Heat plots for expression levels of selected markers were analyzed for the identified subpopulations and are displayed for two animals per group (B-G).

Figure 3: HER2 upregulation leads to activation of NFkB and CCL2 overexpression. (A) Realtime PCR analysis of mammospheres derived from FVB wild type and pre-malignant MMTV-HER2 mammary glands. Mammary glands from FVB Wild type (B), 14 week old (C) and 22 week old (D) MMTV-HER2 mouse mammary glands were stained against CCL2. Signal intensity within the epithelium was quantified (E). Phosphorylation of the p65 subunit of NFkB was analyzed in Western Blots of whole cell lysates of mammospheres from 20 week old pre-malignant MMTV-HER2 mice (F). CCL2 was stained in acini cultures derived from pre-malignant MMTV-HER2 mammary glands that were grown for 5d and then treated with DMSO (vehicle control; G), 1uM Lapatinib (H) or 1uM IKK inhibitor compound A (I) for 24h. Acini derived from 20 week old pre-malignant MMTV-HER2 mouse mammary glands were grown for 5d, then treated with DMSO (vehicle control; J), Lapatinib (1uM; K), IKK inhibitor compound A (1uM; L), or CCR2 inhibitor RS504393 (1uM; M). Macrophages were isolated from 20 week old pre-malignant MMTV-HER2 mammary glands and added. After 24h, acini were stained against F4/80 and the percentage of acini associated with mammary gland macrophages in 3D co-cultures was determined (N). 20 week old pre-malignant MMTV-HER2 mice were treated with a CCR2 inhibitior RS504393 (10mg/g i.p. daily) for 2 weeks. Pre-malignant mammary glands were stained for E-Cadherin and F4/80 (O,P) and the percentage of ducts containing intra-epithelial macrophages (IEM) was quantified (Q).

Figure 4: Intra-epithelial macrophages induce an EMT-like reponse in early cancer cells. 20 week old MMTV-HER2 mice were stained against E-Cadherin and F4/80 and the percentage of individual epithelial cells that showed disrupted E-Cadherin were quantified dependent on whether macrophages did not make direct contact to the duct (A) or whether ducts contain intra-epithelial macrophages (IEM; B) were quantified per animal (C). E-Cadherin mRNA expression by qPCR in whole pre-malignant mammary glands of 20 week old MMTV-HER2 mice was performed (D). The mammary epithelial cell line Comma-1D cells was grown as monolayer and Raw264.7 macrophages stably transfected with mCherry were added. After 12h co-cultures were stained for E-Cadherin (E) or β-Catenin (F) and the signal intensity of β-Catenin signals inside the nucleus was quantified (G). Conditioned medium was harvested from primary cultures of FVB wildtype or pre-malignant MMTV-HER2 mammospheres and added to Raw264.7 macrophages or mammary tissue macrophages isolated from pre-malignant MMTV-HER2 mammary glands. Wnt-1 expression in Raw264.7 macrophages (H) or primary mammary tissue macrophages (MTM; I) was determined respectively. Comma-1D cells were grown as monolayers and Raw-264.7-mCherry macrophages were added alone (K) or together with DKK1 (L). Co-cultures were harvested after 12h and stained against E-Cadherin (J-L). E-Cadherin signal intensity in regions of cell junctions were quantified (M). Sections from human DCIS tissue were stained against CD68 (macrophages) and E-Cadherin (N,O). E-

Cadherin pixel intensity was quantified in regions of cell junctions and compared between different patients with low or high intra-epithelial macrophage numbers (P).

Figure 5: Macrophage depletion during pre-malignant stages prevents early cancer cell dissemination. 20 week old pre-malignant MMTV-HER2 mice were treated with the anti CSF-1Rα ASF98 antibody and animals were harvested after two weeks with no signs of invasive carcinoma. E-Cadherin expression in whole mammary glands was determined by RealTime PCR of whole mammary gland lysates (A) and by immunofluorescent staining against E-Cadherin in mammary gland sections (B). E-Cadherin signal intensity was measured in regions of cell junctions, dependent on the presence of intra-epithelial macrophages (IEM) (D). Pre-malignant mammary glands were stained against cytokeratin (CK) 8/18 and smooth muscle actin (SMA) (E,F) and the percentage of eCCs per duct not covered by myoepithelium was quantified (G). Circulating eCCs were quantified by harvesting peripheral blood and determining the amount of HER2 and CK8/18 positive eCCs per mL blood (H). Each dot represents one animal. Disseminated early cancer cells were quantified by staining lung sections against HER2 (I,K) and quantifying the average of HER2+ cells per 100 fields (K).

Figure 6: Early disseminated cancer cells can contribute to metastasis formation. Sections from DCIS patients were bone marrow aspirates had been analyzed for the presence of DCCs were analyzed blindly. E-Cadherin and macrophages (CD68) were stained and patients were assessed as low risk when they had high E-Cadherin signals and low numbers of intra-epithelial macrophages (IEM) (A) or high risk when E-Cadherin signals were low and IEM numbers were high (B). Imaging were taken with same settings. After unblinding, the positive predictive value was calculated as the number of true positive prediction per positive cases (C). Macrophages were depleted by ASF98 treatment in pre-malignant mice starting at week 18 until mice developed palpable tumors (3mm average) and then discontinued (D). Mice were left until tumors reached a diameter of 1cm and then sacrificed. Solitary DCCs in lung sections (E) and metastases defined as cell clusters bigger than 3 cells (F) were quantified in lung sections stained against for HER2. G: Scheme of macrophage assisted early dissemination during pre-malignant lesions where mammary tissue macrophages (MTM) are recruited by early cancer cells (CC) via HER2 and NFkB mediated upregulation of CCL2. Intra-epithelial macrophages then secrete Wnt proteins and thereby induce an EMT which drives early dissemination. Early disseminated cancer cells then contribute to metastasis formation, either as a slow cycling seeds of metastasis (scenario 1) or by interacting with the microenvironment to make it more permissive for the growth of more adapted late cancer cells (scenario 2).

Supplementary Figure 1: A: List of markers analyzed by mass cytometry (CyTOF). B: Gating scheme to create Visne plots of macrophages and monocytes. C: Analysis of the proportion of monocytes (CD45+/Ly6C+), mammary tissue macrophages (MTM, CD45+/CD64+/CD206+), and tumor associated macrophages (TAM, CD45+/CD64+/VCAM+) per myeloid cells (CD11b and/or CD11c).

Supplementary Figure 2: Visne plots as presented in Fig.2 and (A) and the expression levels of CD206 (B), VCAM1 (C), and Tie2 (D) within the corresponding populations defined by the Visne plots.

Supplementary Figure 3: Overview of Realtime PCR expression analysis of selected cytokines in mammospheres derived from 20 week old MMTV-Her2 mammary glands that were not detectable (n.d.) or did not change significantly (n.s.) in relation to expression levels in FVB wild type mammary glands.

Supplementary Figure 4: A: Wnt ligand expression by Raw264.7 macrophages that were not detectable (n.d.) or did not change significantly (n.s.) after treatment with conditioned medium from FVB or MMTV-HER2 mammary gland mammospheres. B,C: Two individual ducts in a DCIS lesion of the same patient with either high E-Cadherin and low intra-epithelial macrophage (IEM) levels (B) or low E-Cadherin levels and high IEM levels (C) within the same patient. Images were taken with the same settings. D: Quantification of E-Cadherin signal intensity in the region of cell junctions within ducts of the same patients with either high or low numbers of IEMs.

Supplementary Figure 5: A; Experimental setup of macrophage depletion from pre-malignant MMTV-Her mice. ASF98 antibody against CSF1Rα was administered i.p. and animals were sacrificed after two weeks before showing any sign of invasive carcinoma. B: Flow analysis of mammary gland tissue demonstrating the depletion of F4/80+/CD11b+ macrophages after 2 weeks of anti CSF1Rα antibody treatment. C: Sections of pre-malignant MMTV-HER2 mammary glands were stained against F4/80 and the percentage of ducts containing intra-epithelial macrophages (IEM) per animal after 2 weeks of macrophage depletion were quantified. D: Hyperplastic ducts were quantified in HE sections of pre-malignant MMTV-HER2 mammary glands.

Supplementary Figure 6: A: Time from beginning of treatment at age week 18 until development of palpable tumors (approximately 3mm in diameter) in pre-malignant MMTV-HER2 mice treated with

antibody against CSF1Rα or vehicle control. B: Time from first detection of palpable tumors (3mm in diameter) until tumors reached a diameter of 1cm after cessation of anti CSF1Rα therapy. B: Flow cytometric analysis of macrophage content (CD45+/CD11b+/F4/80+) in contra-lateral mammary glands of animals with overt tumors at the end of the experiment. Contra-lateral mammary gland in animals with overt tumors (C) as well as invasive tumors (D) were stained against the macrophage marker F4/80. HE staining (F) and staining against E-Cadherin and Endomucin (endothelial cells; G) in sections of invasive tumors that had been treated with an antibody against CSF1Rα or vehicle control during pre-malignant stages.

Supplementary Figure 7: MMTV-HER2 mice had been treated with a CSF1Rα antibody during pre-malignant stages only and therapy was stopped when palpable tumors appeared until overt tumor stages. Lung sections were stained against HER2 and screened for metastases (cluster of HER2+ cell with 3 cells or more). All metastases in one sections for 3 animals of the treated and the control group are shown.

Material and Methods.

Cells and cell culture. Raw264.7 cells expressing mCherry were generated using mCherry lentiviral vectors and maintained in DMEM (Lonza) with 10% FBS and 1% Pen/Strep. Comma-1D cells were maintained in DMEM-F12 medium containing 2%FBS, 1% Pen/Strep, . DKK1 conditioned media was prepared from DKK1 expressing 293T cells and protein concentration was determined using a Bradford assay. 0.5ug/ml DKK1 protein was used for stimulation. For co-culture experiments, Comma-1D cells were seeded on coverslips and after 12h, Raw-264.7-mCherry cells were added after 12h. Co-cultures were fixed in 2% formalin after 12h.

Microscopy. Formalin fixed and paraffin embedded samples were stained as described. Antibodies used were: Her2 (abcam), F4/80 (abcam), E-Cadherin (Becton Dickinson), beta catenin (cell signaling), Cytokeratin 8/18 (Progen, Heidelberg, Germany), . Microscopic analysis was carried out with a Leica xxx widefield microscope using the yyy software or with a Leica xxx confocal microscope using yyy software. For quantification of signal intensity, regions of interest were defined in original tiff files that had been taken under the same exposure time and settings and the mean signal intensity was measured using the Metamorph software.

Realtime PCR analysis. For analysis of wild type mammary epithelial cells or MMTV-Her2 eCCs, mammary epithelial cells were isolated and grown as mammospheres for 5d as described (Cohn et al., 2010). RNA was isolated using Trizol and Realtime PCRs were performed as described (Adam et al., 2009).

Mouse experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Icahn School of Medicine at Mount Sinai protocol 08-0366 and 2014-0190. For macrophage depletion, we administered 3mg of the CSF1R antibody clone ASF98 on day 1 and 1mg on day 7 and weekly thereafter by injection into the tail vein of eighteen week old pre-malignant MMTV-Her2 mice. PBS was used as vehicle control. Treatment lasted 14 days or until tumors were first palpable. ASF98 antibody was a generous gift of Dr. Miriam Merad. For CCR2 blockade, mice were treated with 10mg/kg of RS504393 (Tocris Bioscience) daily by i.p. injections for 14 days.

3D mammary primary epithelial cell cultures. MMTV-HER2 mice were sacrificed using CO_2 at age 14wk, 18-22wk (early pre-maignant cancer lesions) or when overt tumors had formed. Acini cultures were performed as decribed (Cohn et al., 2010; Debnath et al., 2003). 5.0X10^4 eCCs were seeded in in 400ul Assay Medium in 8-well chamber slides coated with 40ul of Matrigel (Corning). Acini formed at an efficiency of around 30 acini/1.0x10^4 MECs plated. For macrophage co-cultures, primary tissue macrophages were added at a ratio of 500/1x104 eCCs to 5d old acini cultures. For inhibitory treatments, 5d old acini cultures were treated for 24h with 1μM Lapatinib (LC Laboratories), 2uM IKK Inhbitor (Basseres et al., 2014)(generous gift from Dr. Albert Baldwin), 1μM CCR2 inhibitor RS504393 (Tocris Bioscience) or DMSO as vehicle control. Cultures were then fixed for immunofluorescence (IF) with 4% PFA.

Sorting of mammary tissue macrophages. MMTV-HER2 mice were sacrificed using CO_2 at age 14wk, 18-22wk (early pre-maignant cancer lesions) or when overt tumors had formed. Whole mammary glands or tumors were digested in Collagenase/BSA at 37°C for 30-45min. Mononuclear cells were enriched in a Percoll gradient and then macrophages were sorted as viable CD45+/Gr1-/CD11b+/F4/80+ cells.

Patient samples. Paraffin embedded sections from patients with DCIS were obtained from the Cancer Biorepository at Icahn School of Medicine at Mount Sinai, New York, NY and from Thomas Karn, University of Frankfurt, Germany. Samples were fully de-identified and obtained with Institutional Review Board approval, which indicated that this work does not meet the definition of human subject research according to the 45 CFR 46 and the Office of Human Subject Research.

Circulating Cancer Cells (CCCs) and Disseminated Cancer Cells (DCCs) detection. For CCC analysis, blood was drawn by cardiac puncture following IACUC protocols. CCCs were purified using negative lineage cell-depletion kit (Milteny), fixed with 3% PFA for 20 min on ice and cytospin preparations were carried out by centrifugation of blood cells at 500 rpm for 3 min using poly-L-lysine-coated slides (Sigma, MO, USA). Cells were stained with anti-CK8/18 or anti-HER2 antibodies in cytospin preparations.

Flow Cytometry.. MMTV-HER2 mice were sacrificed using CO_2 at age 18-22wk (early pre-malignant cancer lesions) or when overt tumors had formed. Whole mammary glands or tumors were digested in Collagenase/BSA at 37°C for 30-45min. Red blood cell lysis buffer was used to remove blood cells, Fc receptors were blocked using CD16/CD32 antibodies and samples were stained using the following antibodies: CD45-PerCPCy5.5 (Biolegend), CD11b-PeCy7 (eBioscience), CD11c-PE (eBioscience), Gr1-AF700 (eBioscience), Tie2-biotin, F4/80-biotin, VCAM-FITC,

CyTOF analysis.

Statistical Analysis. Statistical Analysis was done using Graph Pad Prism Software. Differences were considered significant if *P* values were <0.05. For all cell cultures, one tailed *student t-tests* were performed. For mouse experiments one tailed *Mann-Whitney* tests were used.

Contributions.

N.L. designed and optimized experimental approach and performed *in vitro* and *in vivo* experiments, analysed data and co-wrote the manuscript, A.M. performed CyTOF experiment, N.S. evaluated human DCIS assessment, M.S.S. performed experiments, T.K. and T.F. provided human DCIS sections and analyzed results, M.M. provided general guidance and oversight and co-wrote the manuscript, J.A.A.-G. designed and optimized experimental approach, provided general guidance and oversight, analysed data and co-wrote the manuscript.

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Competing financial interests. The authors declare no competing financial interests.

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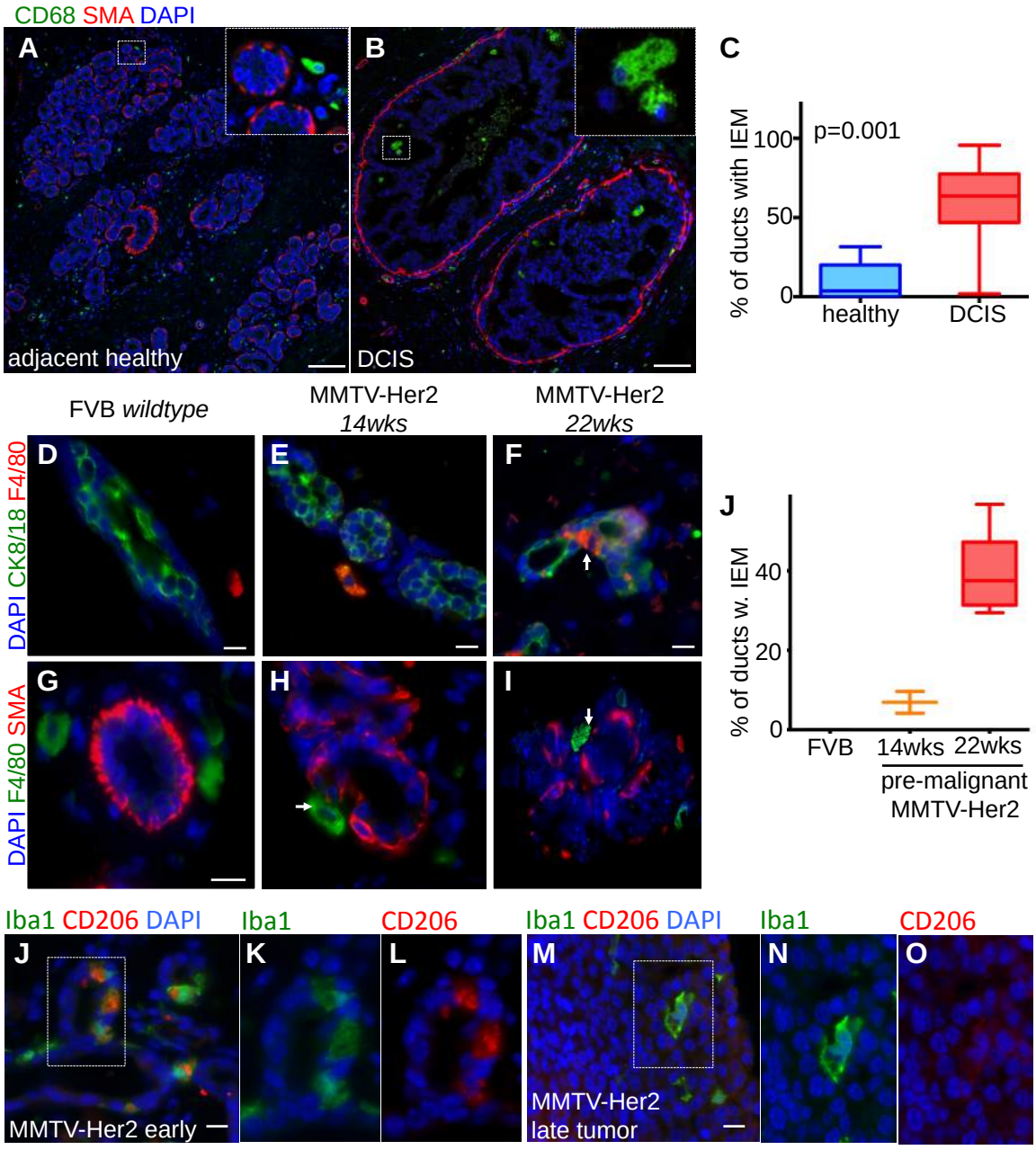
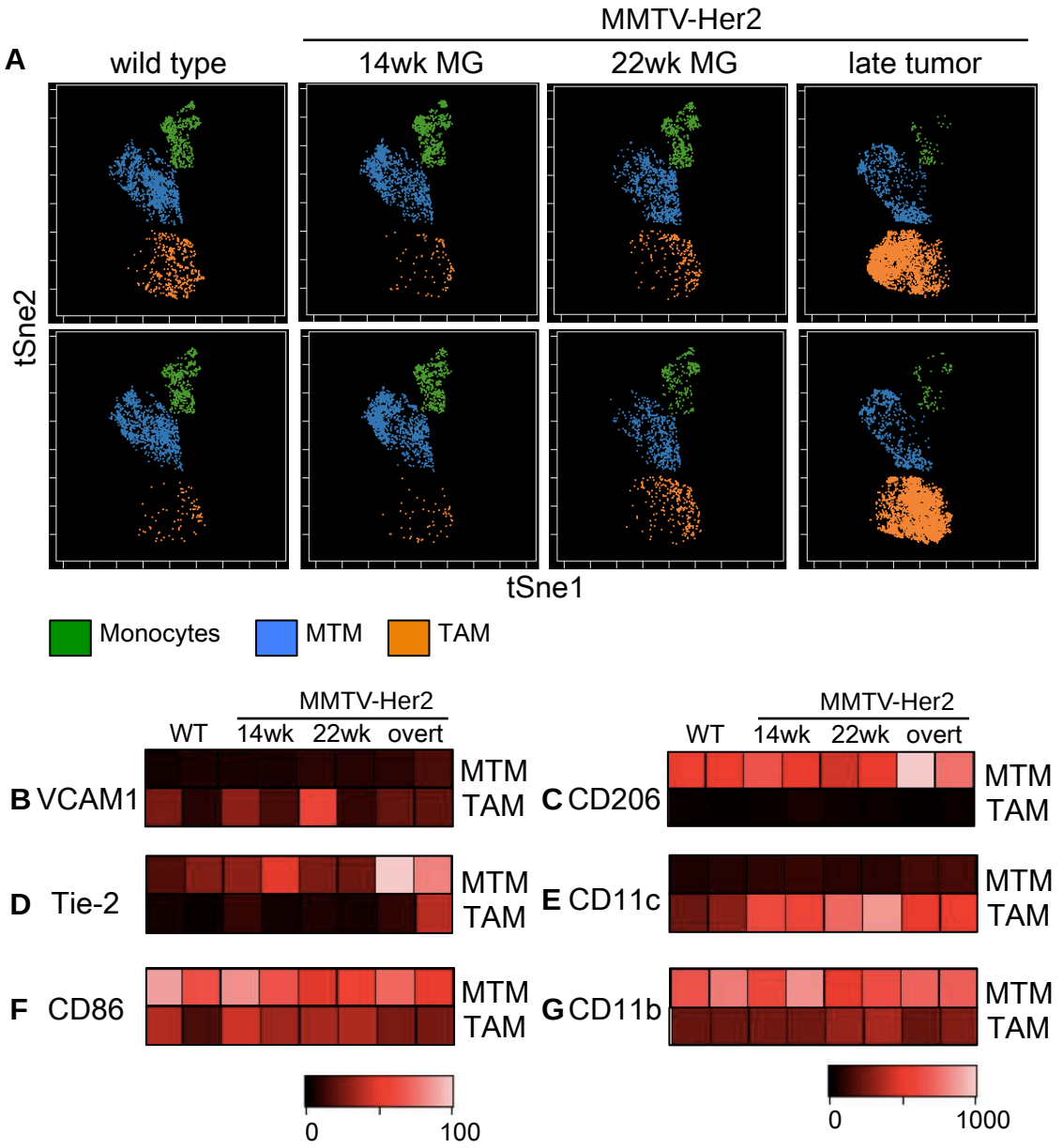
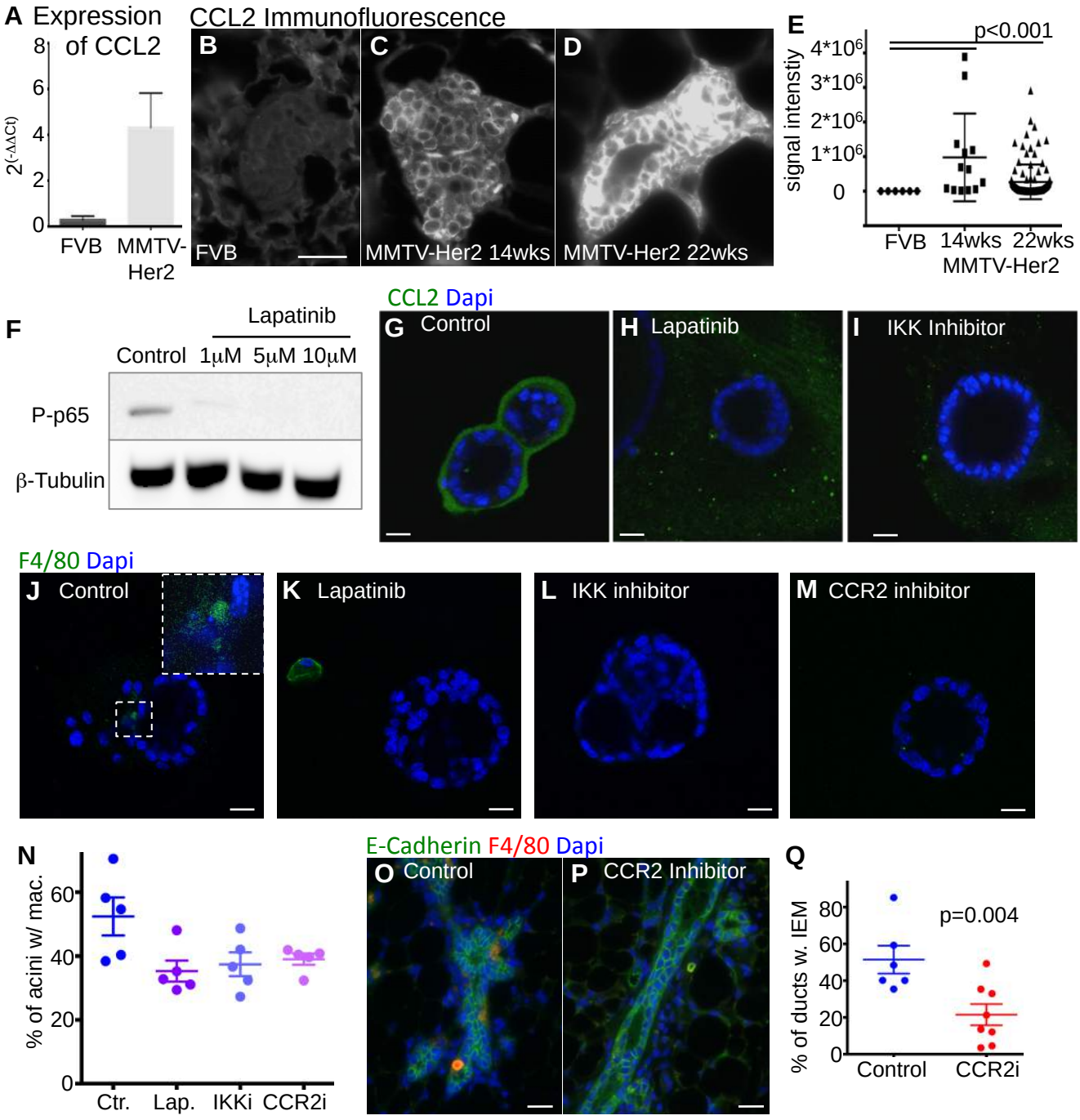


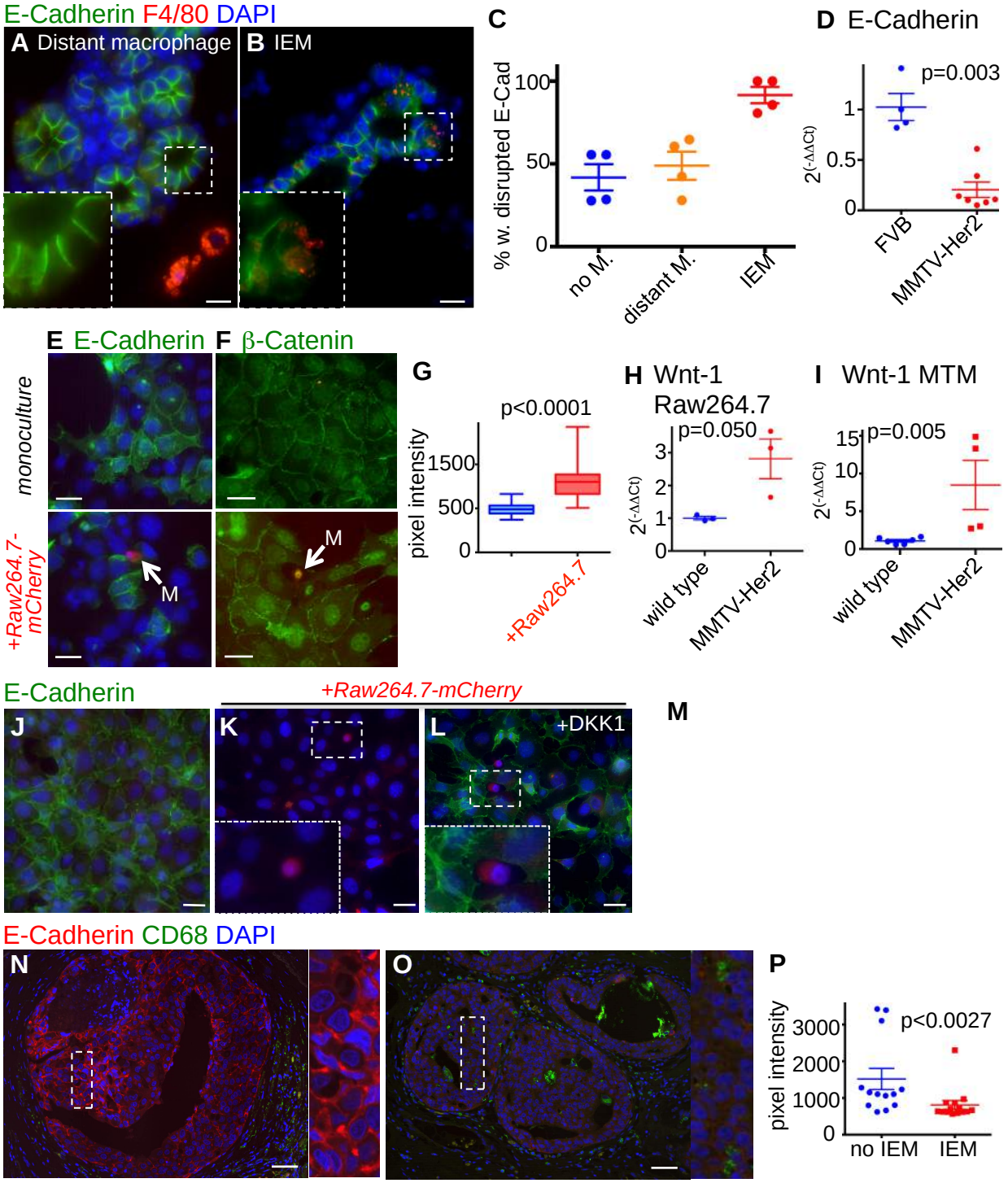
Linde et al. 2015 Figure 1



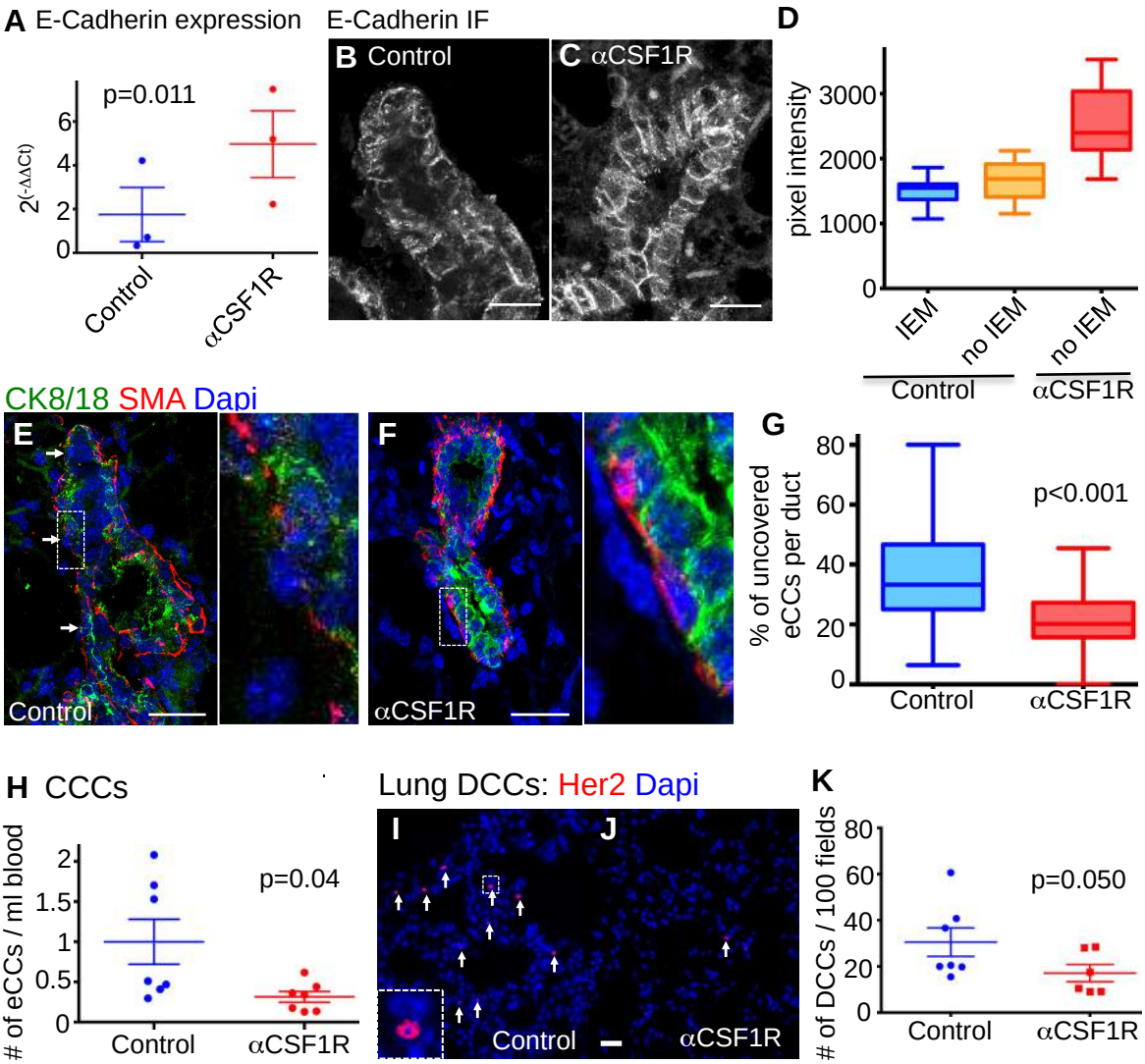
Linde et al. 2015 Figure 2



Linde et al. 2015 Figure 3

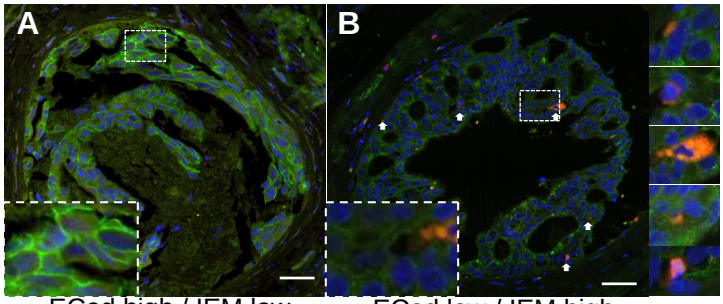


Linde et al. 2015 Figure 4



Linde et al. 2015 Figure 5

E-Cadherin CD68 DAPI

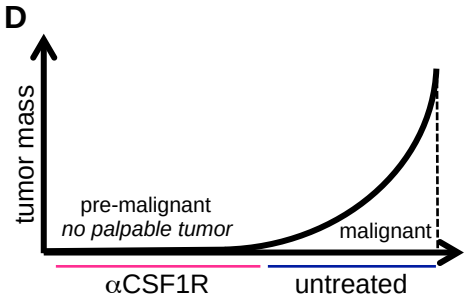


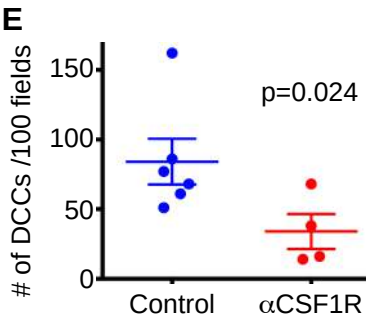
ECad high / IEM low
negative prediction

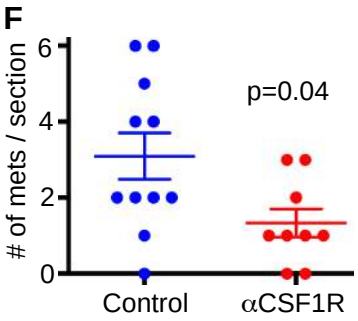
ECad low / IEM high
positive prediction

C

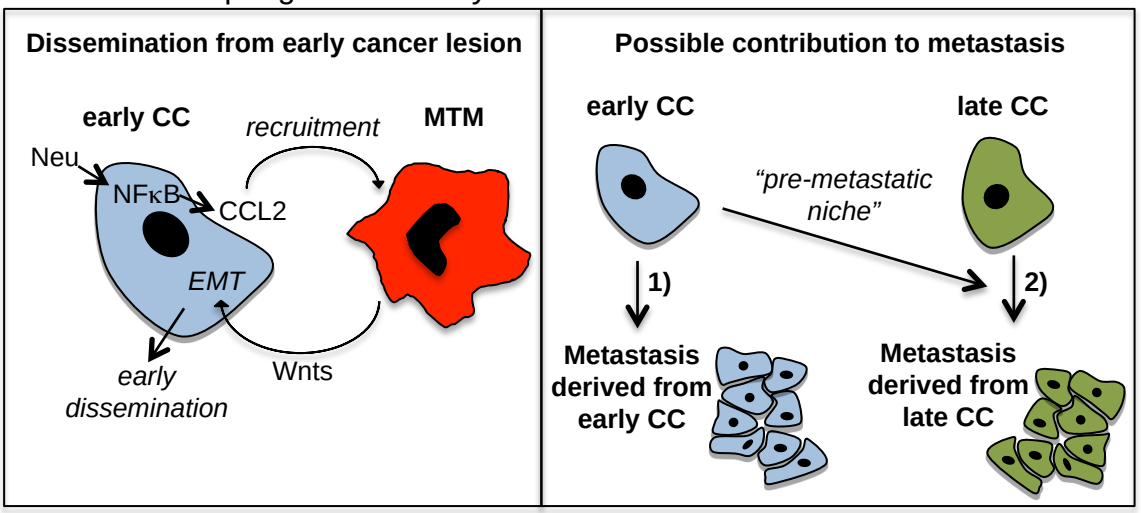
ECad	low	high
IEM	high	low
prediction	positive	negative
# of cases	10	10
Positive for DCCs	7	5
Positive predictive value: 7/10 = 70%		







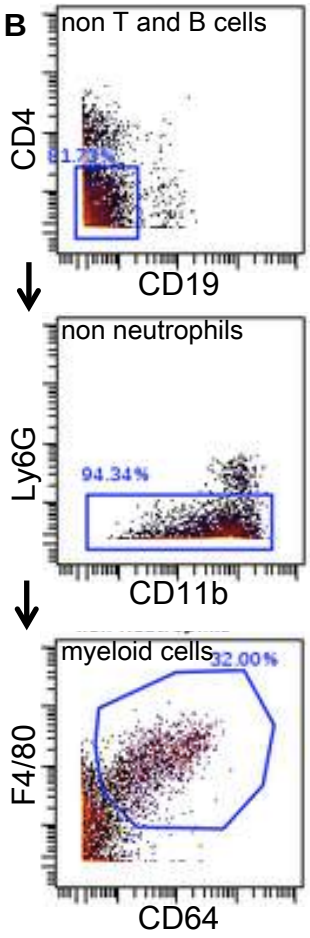
G Model of macrophage driven early dissemination

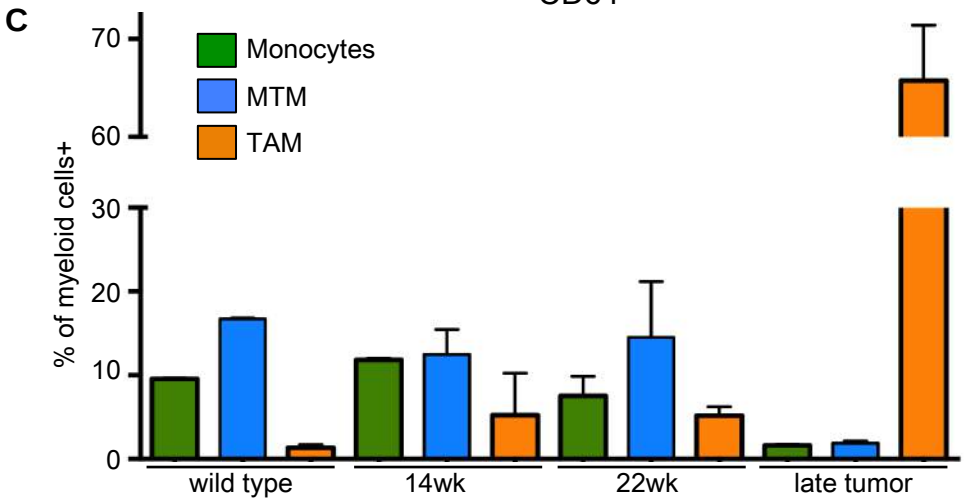


Linde et al. 2015 Figure 6

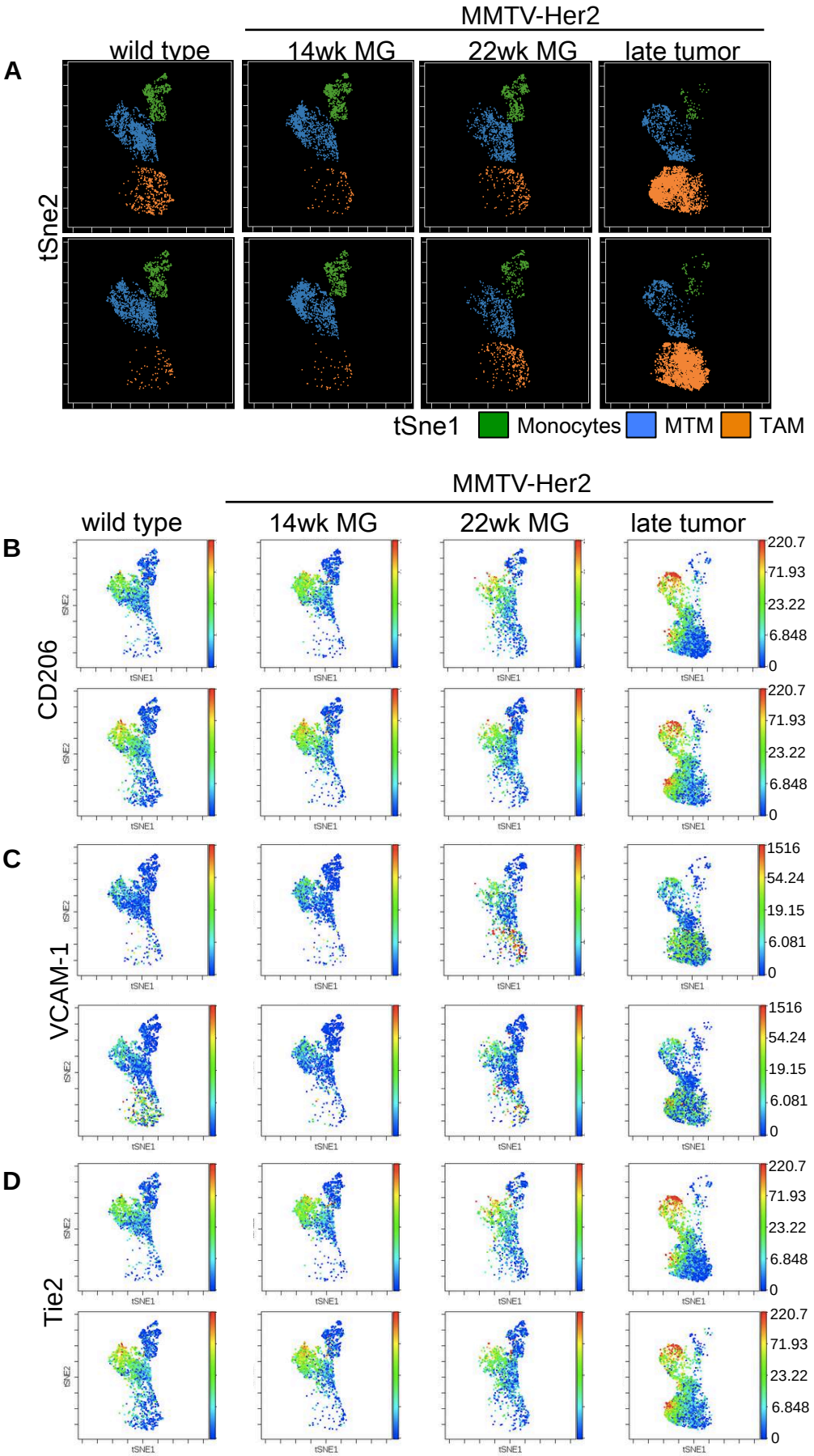
A

Ly6G
CD11c
CD115
CD45
CD11b
CD19
CD24
CD86
CD64
F4/80
FITC-VCAM
Ly6C
APC-CD206
CD103
cKit
CD8
Biotin-Tie2
CD4
MHCII
B220
Cisplatin





Linde et al. 2015 Supplementary Figure 1



Linde et al. 2015 Supplementary Figure 2

A

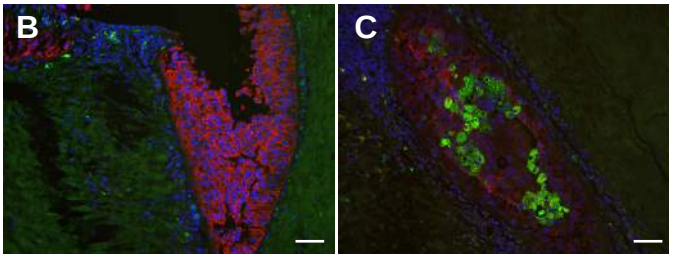
IL1b	n.d.
IL6	n.d.
CSF1	n.s.
CSF2	increased
CCL2	increased

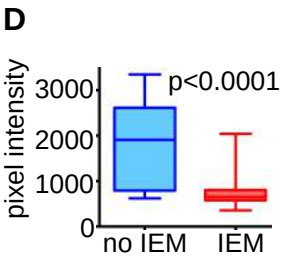
Linde et al. 2015 Supplementary Figure 3

A

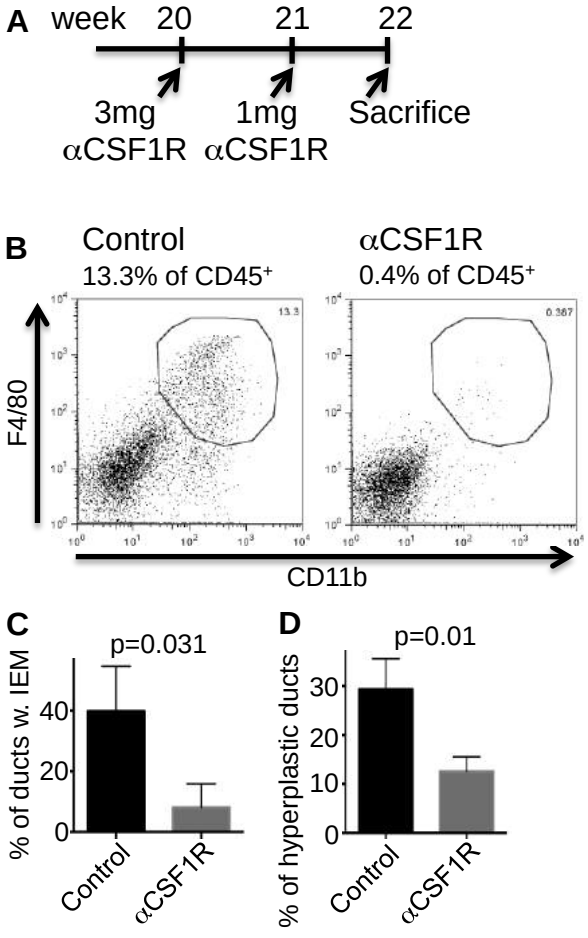
	MTM	Raw264.7
Wnt3a	n.d.	n.s.
Wnt5a	n.d.	n.d.
Wnt7a	n.d.	n.s.

E-Cadherin CD68 DAPI





Linde et al. 2015 Supplementary Figure 4



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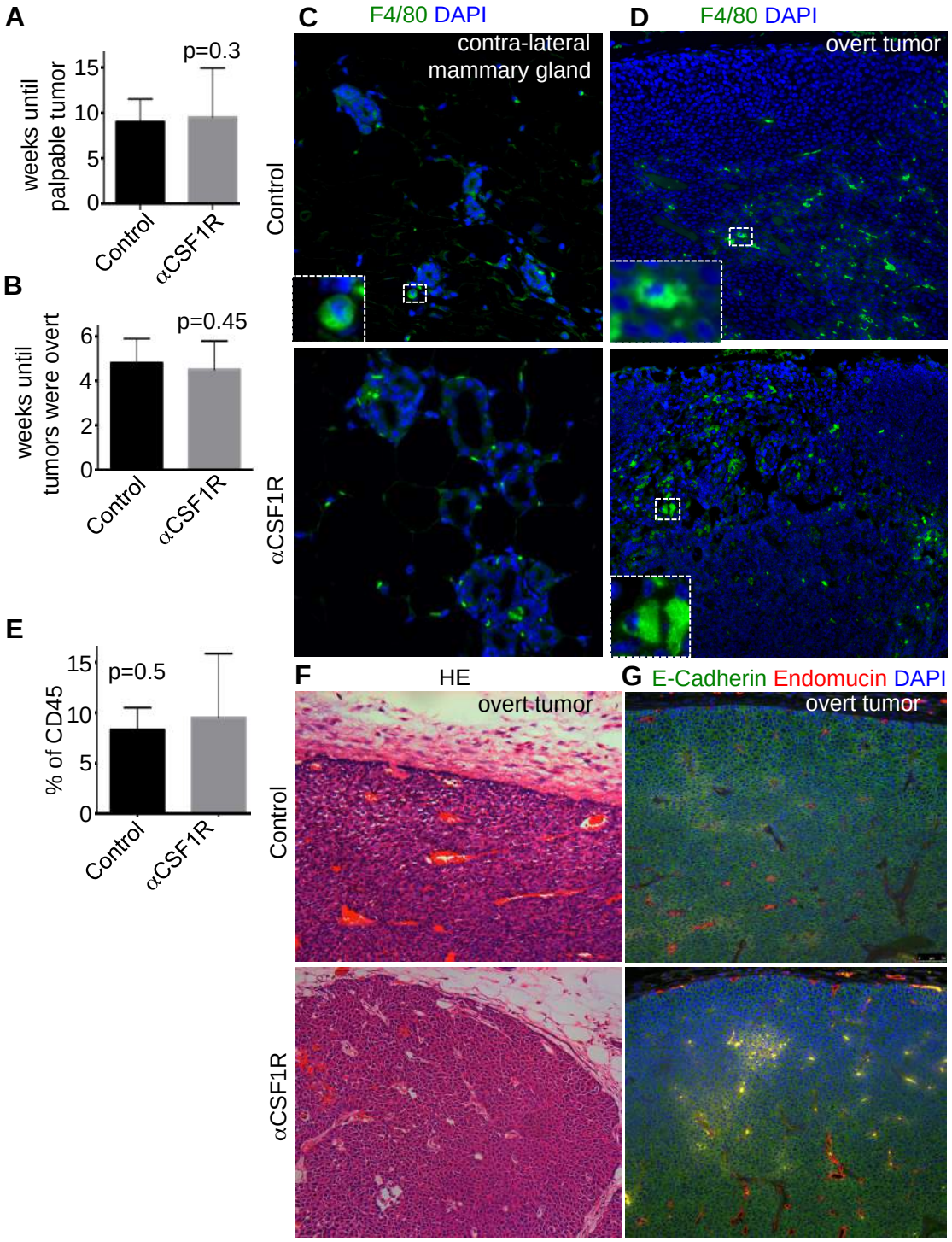

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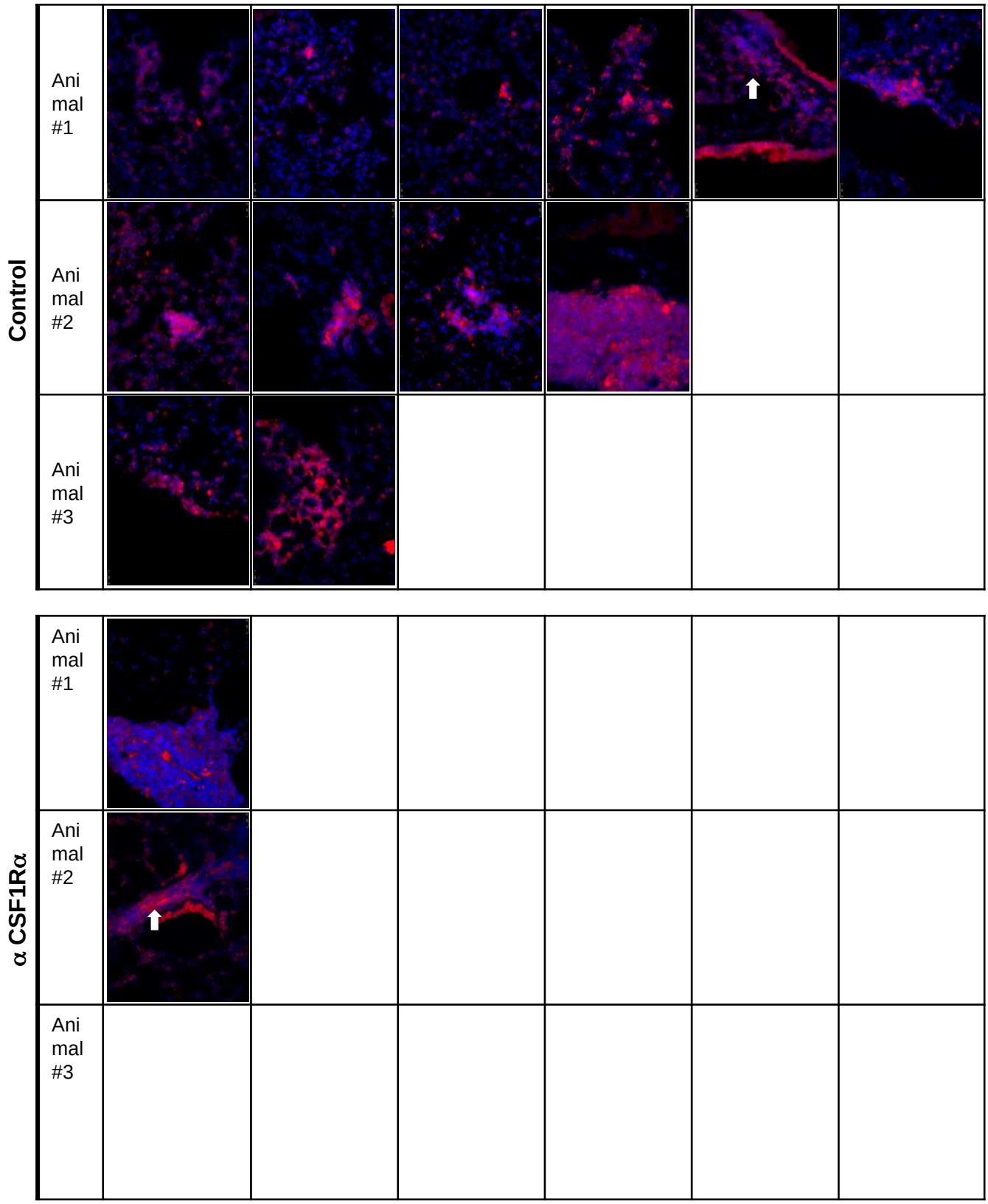

Linde et al. 2015 Supplementary Figure 7